2019

Characterization of IGCR-1: a novel molecule with potential roles in lung carcinogenesis

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

CHARACTERIZATION OF IGCR-1: A NOVEL MOLECULE WITH POTENTIAL ROLES IN LUNG CARCINOGENESIS

by

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B.S., University of California, San Diego 2014

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2019

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ACKNOWLEDGMENTS

I would firstly like to thank Dr. Nader Rahimi for giving me this opportunity to learn, work, and grow as an aspiring researcher in his lab. Your genuine conviction to provide your students with direct mentorship and your passionate pursuit to understand the physical and intangible phenomena of life have taught me lessons that will extend beyond my academic career. Thank you Rachel and Razi for teaching me new lab techniques, helping me with experiments, and giving me valuable advice. Thank you also to all the members in Dr. Rahimi's lab: Evan, Kevin, Shawn, Mohammed, Alvaro, Tooba, Kyle, and Linzi. Thank you for all the fun and thought-provoking conversations during the mornings, afternoons, or nights in lab. Your unique personalities created an enjoyable and supportive atmosphere that made my days in lab memorable. I would also like to thank Dr. Vipul Chitalia for his time and expertise in reviewing and editing my thesis. Finally, I would like to thank my mom, dad, brother, sister, and friends for their faithful care and unending support during this whole process. This work was supported in part through grants from the NIH/NCI (R21CA191970, R21CA193958) and CTSI (1UL1TR001430 to N.R.).

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ABSTRACT

Non-small cell lung cancer (NSCLC) patients respond poorly to generic chemotherapeutics. Despite recent advancements in the treatment of NSCLC, the overall five-year survival rate of NSCLC remains low at 14.6%. We have identified Immunoglobulin-containing and Cysteine-rich Receptor (IGCR-1) as a putative cell surface protein, which is expressed in lung epithelial cells.

The main objectives of this study were to evaluate expression of IGCR-1 in normal lung epithelium and lung carcinoma. Our analysis of publicly available ATCG data via bioGPS revealed that IGCR-1 is highly expressed in human lung. Moreover, our immunohistochemical studies further confirmed that IGCR-1 is expressed in bronchial epithelial cells. Additionally, IGCR-1 is expressed in endothelial cells of blood vessels and localized on the cell surface of human embryonic kidney cells. Its cell surface localization suggests that IGCR-1 could be a cellular adhesion molecule (CAM) with roles in the tumor microenvironment. Of note, the analysis of a panel of human lung carcinoma cell lines via western blot analysis demonstrated that IGCR-1 is expressed at variable levels in these cell lines. Given the heterogeneity of NSCLSs and the corresponding differential expression of IGCR-1 in different lung cancer cell lines, IGCR-1 may play an important role in NSCLCs.

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LIST OF ABBREVIATIONS

CAM ..... Cell Adhesion Molecule

DAPI ..... 4',6-diamidino-2-phenylindole

DMEM ..... Dulbecco's Modified Eagle's Medium

EGFR ..... Epidermal Growth Factor Receptor

IGCR-1 ..... Immunoglobulin-containing Cysteine-rich Receptor-1

IGPR-1 ..... Immunoglobulin-containing Proline-rich Receptor-1

FBS ..... Fetal Bovine Serum

GFP ..... Green Fluorescent Protein

HEK ..... Human Embryonic Kidney Cells

HUVEC ..... Human Umbilical Venous Endothelial Cells

LCK ..... Lymphocyte-specific protein-tyrosine Kinase

MINAR ..... Major Intrinsically-disordered Notch-2 Associated Receptor

NSCLC ..... Non-small Cell Lung Cancer

PAE ..... Porcine Aortic Endothelial Cells

RPMI ..... Roswell Park Memorial Institute Medium

SEER ..... Surveillance, Epidemiology, and End Results

TME ..... Tumor Microenvironment

TMIGD1 ..... Transmembrane and Immunoglobulin domain containing-1

VEGF ..... Vascular Endothelial Growth Factor

INTRODUCTION

Lung cancer is the most commonly diagnosed form of cancer (Street, 2018). However, despite advancement of diagnosis and treatment options, lung cancer remains the leading cause of cancer-related deaths in the United States and worldwide (Siegel, Miller, & Jemal, 2018). The American Cancer Society estimated 154,050 deaths from lung cancer in 2018, garnering a number of deaths greater than those caused by colon, pancreatic, and breast cancer combined (Siegel, Miller, & Jemal, 2018).

Histologically, lung cancers are broadly categorized as non-small cell lung carcinomas (NSCLCs) or small-cell lung carcinomas (SCLCs) (Travis et al, 2011). These two groups are further divided into sub-types based on the anatomical origin of the cancer in the bronchial tree: (A) Squamous cell lung cancers, arising from the main bronchi, (B) Adenocarcinomas, arising from peripheral bronchi, (C) Large cell anaplastic carcinomas, arising from non glandular cells, and (D) NSCLC-not otherwise specified (NSCLC-NOS) (Travis et al, 2011). A general summary of lung carcinoma classification is summarized in Figure 1. NSCLCs represent 75-80% of lung carcinomas and epitomize the heterogeneity of cancers (Testa et al, 2018).

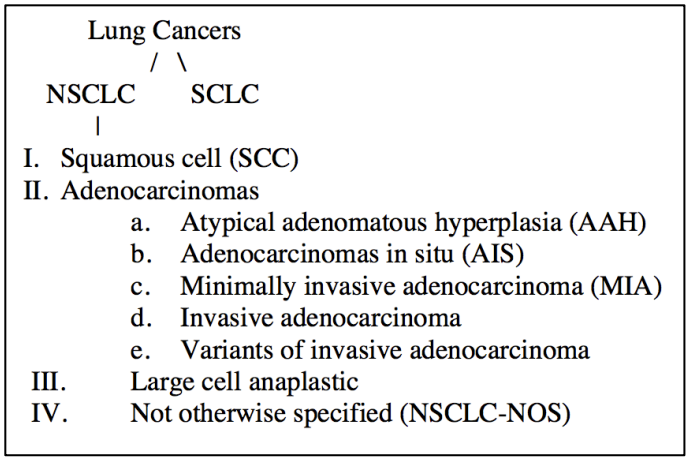


Figure 1: Flowchart of Lung Cancer Classification (Travis et al, 2011).

Molecular Biology of Non-Small Cell Lung Carcinomas (NSCLCs)

Non-small cell lung carcinoma patients manifest a wide range of histologic profiles that stem from each patient case's dynamically different genetic mutation profiles and subsequent variety of phenotypic manifestations (Travis et al, 2011; Balgkouranidou et al, 2013; Pawlikowska et al, 2019). Prior to the understanding of NSCLCs' remarkable genetic and molecular diversity, patients were historically treated with the same cytotoxic chemotherapeutic regimens such as carboplatin or cisplatin (Schiller et al., 2002). Regrettably, patients did not respond well, having poor tumor response rates between 20-30% and median survival duration of 8 months (Schiller et al., 2002; Reck et al, 2016).

In recent years, through the recognition of cell-signaling molecules like epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) as significant players in carcinogenesis, there has been an explosion of new lines of therapy (Dong et al, 2017). Recognition of EGFR as a targetable oncogenic mutation that

drives the malignancy of NSCLCs made precision medicine become more of a reality than a far-off dream (Mok et al, 2009). Targeting specific mutations in cancers as a viable treatment option was first explored and validated by team of researchers in 2011 who showed how targeting the translocation of Philadelphia chromosome chronic myeloid leukemia (CML) significantly improved tumor response rate (Druker et al, 2001). However, despite the initial promise relegated to new target therapeutics to improvie prognosis for NSCLC patients, severe limitations eventually followed (Visconti et al, 2017).

Erlotinib, a EGFR tyrosine kinase inhibitors (TKIs), was first tested for its potential as a standard of care chemotherapy for metastatic NSCLC patients based on the evidence that glycoprotein, EGFR, affects tumorigenesis through clear but complex roles in cell signaling (Shin et al, 1994; Shepherd et al, 2005; Steuer and Ramalingam, 2015). Erlotinib's ability to prolong survival for NSCLC patients, previously treated with first-line or second line chemotherapeutic, led to the development of many more of its kind, such as Gefitinib (Mitsudomi et al, 2010). Although intial groundbreaking studies boasted a longer progression-free survival (median 9.2 vs. 6.3 months) for NSCLC patients with EGFR mutations patients treated with an EGFR TKI (gefitinib) rather than standard chemotherapy, long-term studies showed lower overall survival (12.8 vs 19.8 months) in the gefitinib group (Mitsudomi et al, 2010; Lee et al, 2017).

Because angiogenesis plays a major role in tumor progression, the design of adjuvant therapies to target VEGFRs, whose overexpression had been associated to poor prognosis for NSCLC patients, was also proposed (Han et al, 2001). VEGFR TKIs like

sorafenib and sunitinib target the activating tyrosine kinase domain of VEGFR and have been tested for patients with different cancers including hepatocellular carcinoma, gastrointestinal stromal tumor, renal cell carcinoma, and advanced NSCLCs (Llovet et al, 2007; Escudier et al, 2007; Motzer et al, 2007; Gatzemeier et al, 2006; Socinski et al, 2008). An overall analysis, however, indicates that NSCLC tumor response rates to VEGFR TKIs have remained stubbornly low (8-11%), with a mean progression-free survival no longer than 3.5 months (Subramanian, Morgensztern, and Govindan, 2010).

Targeting oncogenic drivers in NSCLC like EGFR and VEGF have proven to be more effective than standard cytotoxic therapies. However, patients with metastatic NSCLC being treated with these lines of targeted therapies still have a low overall 5-year survival rate at 14.6% (Lin et al, 2016). Despite increased awareness of the toxic effects of smoking, advances in chemotherapeutics based on the identification of growth receptors, NSCLC persists in taking an alarming number of lives (Visconti et al, 2017). These facts could ultimately provoke one to wonder: are there other significant biological players in tumorigenesis that are not being rigorously considered, but should be?

Tumor Microenvironment Giving Clues to Identify New Molecular Targets

NSCLC tumor recurrence, often associated with developed resistance to targeted treatments like EGFR-TKIs, indicate how tumor cells are actively modifying and communicating with their microenvironment to sustain their survival at the face of adversity (Haber et al, 2005; Balkwill, Capasso, and Hagemann, 2012; Hui and Chen, 2015). Studying the tumor microenvironment (TME) consisting of a multitude of

characters including stromal cells, immune cells, fibroblasts, pericytes, adipocytes, blood vessels, extracellular matrix, and cell adhesion molecules would thus provide a different strategy in identifying new molecular targets (Quail and Joyce, 2013). Of particular interest for this study are the roles of cell adhesion molecules (CAMs) and molecules related to the endothelial barrier function. It has been previously shown in breast cancer that a specific CAM, vascular cell adhesion molecule-1 (VCAM-1), is a promising new therapeutic target (Sharma et al, 2017). Similarly in the context of pancreatic cancer, a tumor prone to developing resistance, CAMs have been actively studied to understand its role in the TME (Arcangeli, Crociani, and Bencini, 2014). Recently, the differential interplay between CAMs and immune cells (CD4, CD8 T-cells, B cells, and regulatory T-cells) in NSCLC was described (Chae et al, 2018). This study showed how endothelial adhesion molecules and cellular barrier molecules could be potential biomarkers for immunotherapy due to their distinct roles in recruiting or decreasing immune cell infiltration.

These studies have illustrated the convincing need to understand molecules of the TME in the context of breast and pancreatic cancer. Overall, however, the study of CAMs and the endothelial barrier is still in its early stages, especially in the context of the TME in NSCLCs. Consequently, there is also inadequate translation of research findings to clinical application, leaving patients suffering from recurrent disease with marginal advancement in treatment options.

(A) Cell Adhesion Molecules

Cell adhesion molecules (CAMs), including immunoglobulin superfamily (IgCAMs), integrins, and cadherins, have 2 main functions: (1) mediate cell-cell adhesion in the extracellular matrix, and (2) regulate activity of signaling molecules like receptor tyrosine kinases (RTKs) (Gibson, 2011). In normal development, CAMs are under tight regulation as they are responsible for vital cellular processes such as cell proliferation, migration, and differentiation (Gibson, 2011; Wong, Dye, and Coombe, 2012). Aberrant CAMs are exploited by tumor cells in order to avoid apoptosis, continue proliferation, invade surrounding tissue, and eventually metastasize (Wong, Dye, and Coombe, 2012; Hanahan and Weinberg, 2011). Despite their direct linkage to tumor metastasis, the full range of CAMs' functions are still poorly understood. Hence, their potential as biomarkers or more effective treatment targets has not yet been fully leveraged.

(1) N-cadherin and the Epithelial-Mesenchymal Transition

The hallmark of tumor metastasis is the epithelial-mesenchymal transition (EMT), in which normal epithelial cells lose their non-migratory nature, obtain mesenchymal phenotype, and the gain the ability to initiate immunosuppression, escape senescence, and effectively spread (Mrozik et al, 2018; Thiery et al, 2009; Gheldof and Berx, 2013). An example of a CAM playing a role in modulating tumor metastasis in the EMT is neural (N)-cadherin (Mrozik et al, 2018). During the EMT, epithelial (E)-cadherin loses expression and triggers the upregulated expression of N-cadherin. N-cadherin, unlike E-cadherin, enhances the migratory and invasive behavior of tumor cells (Wheelock et al, 2008). This phenomenon called the E-cadherin to N-cadherin switch currently serves as

an important biomarker for prostate cancer (Gravdal et al, 2007). Like N-cadherin, there may be many other CAMs whose upregulated or downregulated expression may imply its role in the metastasis of tumors.

Novel Cell Adhesion Molecules Showing Evidence of Regulatory Roles in the TME

Through screening the human genome for immunoglobulin (Ig)-containing proteins not yet identified, our lab found a family of novel molecules including: Immunoglobulin-Containing and Proline-Rich Receptor-1 (IGPR-1), Transmembrane and Immunoglobulin Domain-Containing 1 (TMIGD1), and Immunoglobulin-Containing and Cysteine-Rich receptor (IGCR-1). IGPR-1 is a 55kDa protein with an extracellular immunoglobulin domain, transmembrane domain, and proline-rich cytoplasmic domain, shown to play significant cell signaling roles in colon cancer (Rahimi et al, 2012). TMIGD1 is a highly conserved molecule in humans and other species, has been shown to play a regulatory in kidney cancers. (Arafa et al, 2015). IGCR-1, the third one of its family, has a unique cysteine-rich cytoplasmic domain that may contribute to its own characteristic regulatory roles in human cancers.

(A) IGPR-1 Inhibits Colon Cancer Growth and Improves Chemotherapy

Expressed exclusively in eukaryotes, IGPR-1 is the first novel cell adhesion receptor in its family to be identified and characterized (Rahimi et al, 2012). Although it is located in various human organs and tissues, IGPR-1 is found predominantly in epithelial and endothelial cells, functioning to regulate cellular morphology, focal adhesion, and angiogenesis (Rahimi et al, 2012). The identification of IGPR-1's functions

are derived from its proline-rich cytoplasmic motifs found to be associated with Src homolog 3 (SH3) protein which interacts with Nck (SPIN90), a known molecule that regulates cellular adhesion by reorganizing actin cytoskeleton (Kaneko et al, 2008; Rahimi et al, 2012; Lim et al., 2001; Takenawa and Miki, 2001)

IGPR-1 plays a role in modulating angiogenesis: an essential biological process that is starkly hijacked by tumor cells to advance their uncontrollable proliferation (Folkman, 1996; Holash et al, 1999, Leenders et al, 2002, Rahimi et al, 2012). Tumors become a garden of flourishing malfunctioned blood vessels n this process of pathogenic angiogenesis, causing the integrity of the endothelial barrier to be jeopardized (Baluk et al, 2005). Existing in cis-and trans-dimeric forms, IGPR-1 effectively regulates angiogenesis and the endothelial barrier adhesion function through trans-homophilic dimerization (Figure 2).

In colorectal cancer (CRC), one of the United States' most common malignancies and the third most commonly diagnosed cancer, IGPR-1 is found to induce multicellular aggregation of colon tumor cells and increase their resistance to chemotherapeutics like doxorubicin (Siegal, 2017; Woolf et al, 2017). The effects of IGPR-1 decreasing the efficacy of well-known cytotoxic drugs such as doxorubicin make IGPR-1 a novel chemotherapeutic target (Woolf et al, 2017). These findings validate how CAMs play a crucial role in the tumor microenvironment.

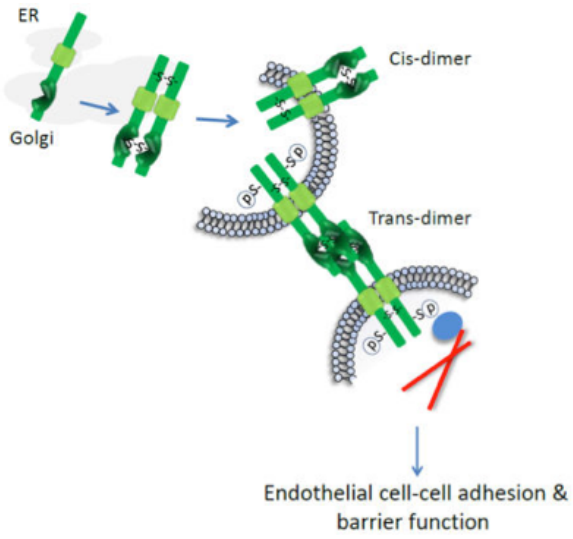


Figure 2: Proposed Mechanism of IGPR-1's Function. IGPR-1 regulates angiogenesis and the endothelial barrier function through trans-homophilic dimerization (Wang et al, 2016).

(B) TMIGDI: a Tumor Suppressor in Renal Cancer

Acute kidney injury (AKI) and chronic kidney disease (CKD) are interrelated diseases with high mortality and morbidity rates, yet the underlying mechanisms of these diseases are still poorly understood (Chawla et al, 2014; Okusa, Chertow, and Portilla, 2009). In 2015, TMIGD1, the second novel cell adhesion molecule in the IGPR family, was discovered to play significant regulatory and protective roles in renal tubular epithelial cells (Arafa et al, 2015). It regulates cell migration and cell morphology, as well as protects renal epithelial cells from damage caused by oxidative stress and nutrient deprivation (Arafa et al, 2015). With the identification of TMIGD1 as a novel cell adhesion molecule that protects renal epithelial cells from becoming susceptible to AKI

and CKD, the question of its potential role in renal cell carcinoma (RCC) naturally surfaced and sought to be answered.

In human RCCs, TMIGD1 acts as a tumor suppressor (Meyer et al, 2017). This novel molecule showed evidence of regulating the induction of well-known cell cycle inhibitors: p21CIP1 and p27KIP1 (Meyer et al, 2017). Its expression is downregulated in human RCCs due to its direct relationship with downregulated transcription factor regulator CCAAT/enhancer-binding proteinβ (C/EBPβ) observed in renal cancers (Meyer et al, 2017; Nerlov, 2007). Interestingly enough, over-expressed TMIGD1 in renal tumor cells showed significant tumor growth arrest and decreased tumor metastatic activities such as morphogenic branching and cell migration. The discovery of TMIGD1 and the characterizing studies that elucidated its function affords researchers and clinicians a new angle of approach to not only better understand the pathobiology of RCC, but also to design more effective therapies for renal cancer patients.

(C) Immunoglobulin-Containing Cysteine Rich (IGCR-1) is expressed in Lung Epithelial Cells: A Potential Role for IGCR1 in Lung Carcinoma

In a similar manner, IGCR-1 is now being characterized to understand its regulatory role in the context of NSCLCs tumorigenesis. Immunoglobulin-Containing and Cysteine-Rich receptor (IGCR-1) was originally identified as one of 386 putative uncharacterized cell-surface proteins based on a full analysis of the human genome along with IGPR-1, TMIGDI, and MINAR1 (Rahimi et al, 2012; Arafa et al, 2015; Ho et al, 2018). Composed of 407 amino acids, IGCR-1 is predicted to have a molecular weight of 45kDa (unpublished data). *In silico* analysis suggested that IGCR-1 is a cell surface

molecule with three characteristic domains: 1) two immunoglobulin domains, 2) a transmembrane domain, and 3) a cysteine-rich cytoplasmic domain. IGCR-1 was determined to be highly expressed in lung epithelial cells and predicted to have roles in regulating lung carcinoma.

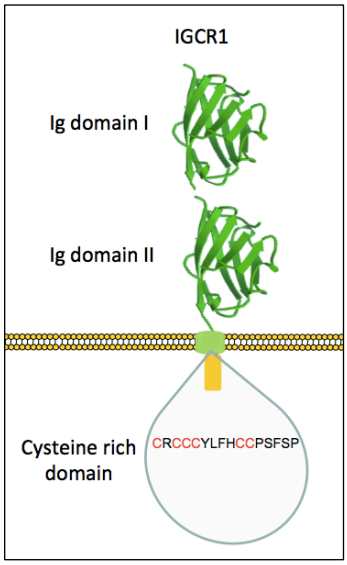


Figure 3: Proposed Structure of IGCR-1. The structure of putative molecule, IGCR-1, has three characteristic domains: (1) two immunoglobulin domains, (2) one transmembrane domain, and (3) a cysteine-rich cytoplasmic domain (unpublished data).

The overall goal of this project was to examine expression of IGCR-1 in lung carcinoma and specifically test the hypothesis that IGCR-1 is differentially expressed in human lung carcinoma.

The specific aims of this project are as follows:

I. Determine the expression of IGCR-1 in normal lung tissue, identify the cell types that are positive for IGCR-1, and test the hypothesis of IGCR-1 as a cell surface molecule.

II. Determine expression of IGCR-1 in human lung cancer cell lines.

III. Determine expression status of IGCR-1 in different staged human lung carcinomas and identify the tumor and/or stromal cell types in the tumor microenvironment that are positive for IGCR-1.

METHODS

Antibodies and General Reagents

Rabbit polyclonal anti-IGCR-1 antibody was developed as previously described (Rahimi et al, 2012). Anti-IGCR-1 antibody was used in 1:2000 dilutions for western blot analysis and immunofluorescence analysis. 1:5000 dilutions of anti-IGCR-1 antibody was used for immunohistochemical analysis of the panel of human lung cancer samples purchased from US Biomax, but 1:2000 dilutions were used for all other immunohistochemistry experiments. Mouse monoclonal anti-GAPDH antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and used at 1:1000 dilutions. Immunohistochemistry staining kit including peroxidase and protein blocking reagents, biotinylated secondary antibody, streptavidin-Horseradish peroxidase (HRP), and 3,3'-diaminobenzidine (DAB) substrate was purchased from Abcam. Hematoxylin was purchased from Thermo Scientific™.

Cell Culture

Human embryonic kidney cell line (HEK-293), HEK-IGCR-1, and 293-GPG cells were grown in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS). Lung cancer cell lines including A549, H1299, H3030, and PC-9 were maintained in Roswell Park Memorial Institute Medium (RPMI). All cell lines were kept at 37˚C and 5% CO_2 conditions by Thermo Scientific humidified CO_2 incubators.

Retroviral Virus Production

pMSV.puro vector containing IGCR-1 sequence and cMyc tag was transfected into 70-80% confluent human 293-derived retroviral packaging cell line (293-GPG) grown in GPG growth media (DMEM, 10% FBS, 2mM L-glutamine, and 50 units/mL penicillin and streptomycin). After 24-hour incubation, virus-producing media replaced GPG growth media. Viral supernatant was collected over 3 days, at 72, 96, and 120 hours (Rahimi et al, 2000). HEK-293 cells expressing IGCR-1 were produced using this retroviral system. DMEM containing 10% FBS and 1µg/mL puromycin was used to select for successfully transduced cells.

Immunohistochemical Analysis

Wild-type mice were subjected to organ extraction. Isolated organs were subjected to standard procedures of formalin fixation and paraffin embedding (FFPE). Tissue sections were prepared with Microtome (Model HM 325) and Boekel Lighted Tissue Flotation Bath III (Model 145702). After baking overnight at 60˚C, tissue slides underwent hydration steps with Pro-Par clearant. Samples were placed in citrate buffer at pH 6.0 and heat-induced epitope retrieval steps were achieved through the BioCare Medical Decloaking chamber. Primary antibodies were diluted into serum-free protein block overnight at 4˚C to 1:2000 or 1:5000 dilutions of anti-IGCR-1 rabbit polyclonal antibody. Antibody detection was preformed using 30-minute incubation with anti-rabbit-HRP. Washes were performed with 0.05% tris-buffered saline polysorbate (TBS Tween). Slides were subjected to 40 seconds of DAB chromagen and 40 seconds in Hematoxylin.

After dehydration, slides were mounted with VectaMount® Permanent Mounting Medium.

Human breast cancer and leiomyosarcoma tissue samples were provided by Boston Medical Center with IRB approval. Lung carcinoma progression tissue microarray (LC1005a) containing 15 cases of squamous cell carcinoma, 15 adenocarcinoma, 8 large cell carcinoma, 8 lymph node metastasis carcinoma, 46 matched or unmatched adjacent normal tissue, plus 8 normal lung tissue was purchased from US Biomax, Inc.

Western Blot Analysis

Cell lysates were made when cells were 70-90% confluent. Two washes with H/S buffer (25mM Hepes (pH 7.4)/150mM NaCl) and aspiration were performed to remove excess media and cellular debris. Lysis buffer (10mM Tris-HCl, 10% Glycerol, 5 mM EDTA (pH 7.4), 50 mM NaCl, 50 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, 15µL phosphatase inhibitor, 10µL $NaVIO_4$) was used. Samples were subjected to centrifugation at 15,000 rpm for 15 minutes at 4˚C. After collection of supernatant, 5X sample buffer (3.8% Tris-base, 50% glycerol, 5% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 0.0025% bromophenol blue) was added to samples and subsequently vortexed and heated at 95-100˚C for 3-5 minutes.

12% SDS-polyacrylimide resolving gel (SDS-PAGE) and 2X stacking gel were made. Samples were subjected to 35mA for 30-60 minutes and to 45mA for 4-5 hours

until significant separation between 43kDa and 55kDa bands as indicated by EZ-Run™ prestained *rec*-protein ladder purchased from Fisher BioReagents™. Proteins were transfered onto polyvinlidene difluoride (PVDF) membranes. Membranes were incubated in blotto (2% non-fat dry milk and 0.05% Tween-20 in Western Rinse) for 1 hour at room temperature or overnight at 4˚C. 1:10,000 dilutions of secondary rabbit or mouse were used. Membranes were developed using enhanced chemiluminescence (ECL).

Immunofluorescence Microscopy

An estimated 7.5-8.5 x 10^4 cells were seeded in 6cm plates. Ethanol and 15 minutes of ultraviolet germicidal irradiation were utilized for sterilization. HEK-293 cells were transfected with E-cadherin GFP and grown to become 80-90% confluent. Cells were fixed in 4% paraformaldehyde for 15 minutes and subjected to permeabilization with 0.1% sodium citrate and 0.1% Triton X-100. Samples were incubated for one-hour with anti-IGCR-1 antibody (1:2000) and subsequently incubated for one-hour with anti-rabbit secondary antibody (1:750). Three washes with non-sterile phosphate buffered saline at five minutes each were performed before and after primary and secondary incubation steps. After mounting, samples were visualized under immunofluorescence microscope.

RESULTS

IGCR-1 is highly expressed in specific cell types of normal lung tissue

We began our study with a preliminary screening of a panel of cell types from major organs to predict IGCR-1 expression levels by its transcript frequency in different cell types using publicly available ATCG data via bioGPS. From this analysis, we found that the IGCR-1 transcript was most prevalent in bronchial and airway epithelial cells, suggesting higher expression levels in these cell types (Figure 4). Albeit at lower levels, IGCR-1 transcript was also detected in a variety of other cell types including ones of the nasopharyngeal epithelium, medial temporal gyrus, and plasma (Figure 4).

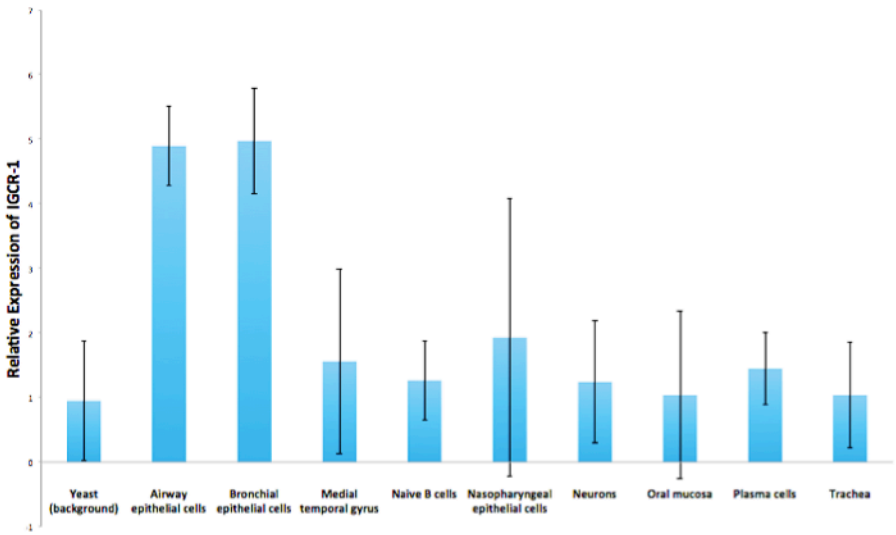
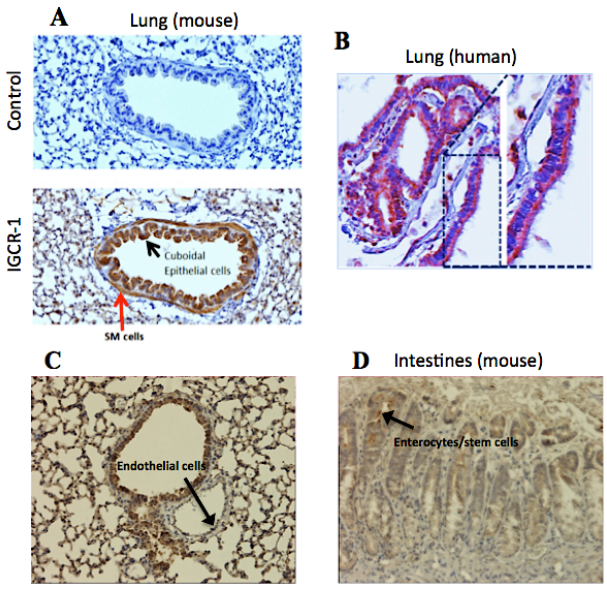


Figure 4: IGCR-1's predicted expression across a panel of cell types using bioGPS database. Preliminary screening of IGCR-1 transcript levels suggested that IGCR-1 is highly expressed in lung bronchial and airway epithelial cells relative to other cell types.

To validate this finding, we continued our study with immunohistochemistry (IHC) staining of a mouse tissue array to determine whether IGCR-1 would be more highly expressed in lung epithelial cells than other cell types and tissues. Indeed, lung tissues showed the strongest indication of IGCR-1 expression (Figure 5e). Additionally, compared to the control, cuboidal shaped ciliated bronchiole epithelial cells indicated the highest intensity staining of IGCR-1, specifically in certain apical regions (Figure 5a). These findings were consistent in human lung tissue (Figure 5b). Smooth muscle cells, endothelial cells, and apical regions of enterocytes also expressed IGCR-1 (Figure 5a, c-d), but at lower levels compared to lung bronchial epithelial cells (Figure 5e). Cell types of the eye, skin, or brain did not show significant expression of IGCR-1 (Figure 5e).



E

Tissue	Cell Type	Level of Expression
Lung	Club Cells	++
Intestines	Enterocytes; Stem Cells	+
Blood Vessels	Endothelial Cells	+
Smooth Muscle	Smooth Muscle Cells	+
Eye	all	low
Skin	all	low
Brain	all	low

Figure 5: IGCR-1's expression across a panel of cell types and specific cell-types expressing high levels of IGCR-1 from immunohistochemical (IHC) analyses. (A)

IGCR-1 is expressed in mouse ciliated bronchial epithelial cell types with higher intensity in select apical regions and at lower intensity in smooth muscle cells. (B) Similar IGCR-1 expression profile in human lung tissue. (C-D) IGCR-1 is expressed in endothelial cells and enterocytes with less staining intensity. (E) Summarized indications of IHC analyses showing relatively insignificant level of IGCR-1 expression observed in eye, skin, and brain tissues. Slides were visualized in light microscope at 20X.

IGCR-1 is a cell-surface molecule

To further characterize this novel molecule, we wanted to investigate its cellular localization. From its putative amino acid sequence, IGCR-1 was predicted to be a cell surface receptor with two immunoglobulin domains, a transmembrane domain, and a cysteine-rich cytoplasmic domain (Figure 3). Our initial results using immunofluorescence indicate that IGCR-1 is localized on the cell surface of human embryonic kidney 293 cells (HEK-293), having insignificant overlapping expression with the control staining cellular nuclei (Figure 6). Western blot analysis was done with GAPDH loading control to validate IGCR-1 expression in HEK-293 cells (Figure 6).

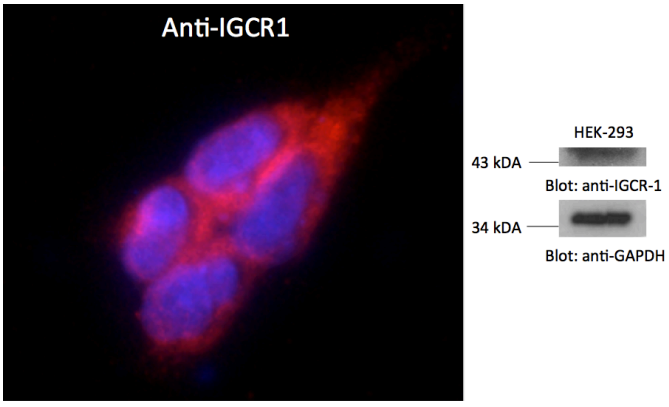


Figure 6: Preliminary results testing hypothesis of IGCR-1 as a cell surface molecule using immunofluorescence microscopy. HEK-293 cells show IGCR-1 (Texas red) localized on the cellular surface. Control with DAPI (blue) stained for nuclei. Slides were

visualized under immunofluorescence microscope at 40X. Expression of IGCR-1 in HEK-293 cells is verified using western blot analysis and GAPDH as loading control.

Differential expression of IGCR-1 in cancer cell lines

To evaluate expression of IGCR-1 in human lung cancer cell lines, we used a panel of cancer cell lines and found that IGCR-1 was variably expressed in different human cancers (Figure 7). Among nine cancer cell lines, four cell lines consisting of breast carcinoma (BT-20), colorectal adenocarcinoma (HT-29), amelanotic skin melanoma (A-375), and skin melanoma (UACC-62) showed the greatest protein levels of IGCR-1 (Figure 7). The remaining cancer cell lines did not have significant levels of IGCR-1 compared with GAPDH loading control (Figure 7). Interestingly, differential expression of IGCR-1 was also observed within cancer cell lines of the same organs. For example, although colorectal adenocarcinoma (HT-29) showed positive expression of IGCR-1, other cancer cell lines of the kidney such as kidney carcinoma (A-498), clear cell renal cell carcinoma (CAK1-1), colorectal carcinoma (HCT 116), and renal cell adenocarcinoma (786-0) did not indicate having significant protein levels of IGCR-1 (Figure 7). In the same way, for skin cancers, malignant skin melanoma (SK-MEL-2) did not have indications for positive IGCR-1 expression despite positivity observed in amelanotic skin melanoma (A-375) and skin melanoma cell lines (UACC-62) (Figure 7).

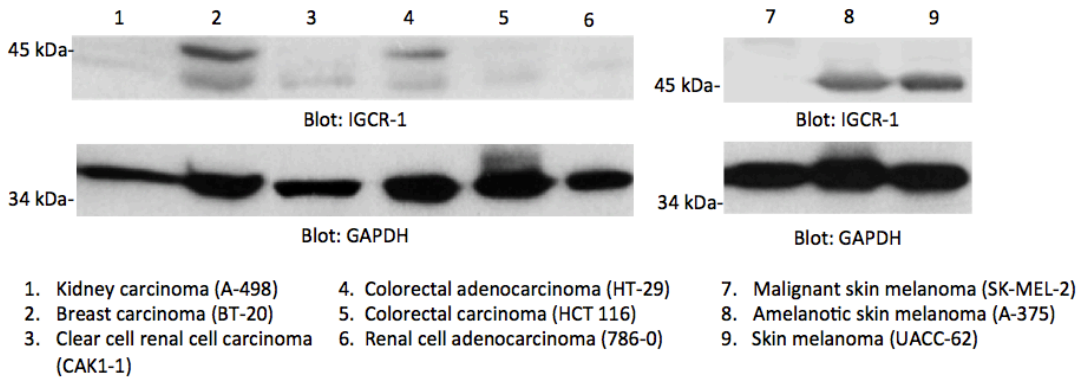


Figure 7: Western blot analyses to determine specific cancer cell lines expressing IGCR-1. Breast carcinoma (BT-20), colorectal carcinoma (HCT 116), amelanotic skin melanoma (A-375), and skin melanoma (UACC-62) cell lines indicated positive IGCR-1 protein levels compared to GAPDH loading control.

IGCR-1 is downregulated in breast cancer and leiomyosarcoma

To elucidate its role in cancer biology, we continued our analysis by doing IHC comparison studies to observe whether or not IGCR-1 expression levels differed in tumor cell types compared with normal cell types. We performed IHC analysis on five different breast cancer patient cases. Normal ductal breast cell types stained much more intensely than tumor ductal cell types (Figure 8a, Case 1). Areas of metastasis showed even lower expression levels of IGCR-1, consistent with evidence of IGCR-1 downregulation in tumorigenesis (Figure 8a, Case 2). To test whether this trend is maintained in other cancer types, nine smooth muscle tumor (leiomyosarcoma) patient cases also underwent IHC staining to probe for IGCR-1. Again, when compared with normal smooth muscle cells of large arteries, smooth muscle cells of leiomyosarcomas showed evidence of IGCR-1 downregulation (Figure 8b).

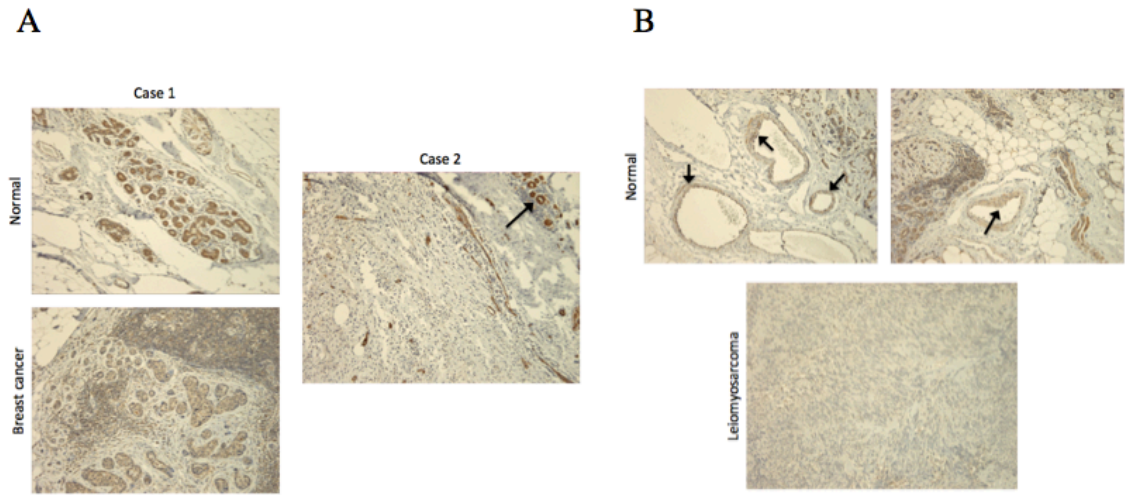


Figure 8: IHC comparison analyses of human breast cancers and leiomyosarcoma. (A) Case 1: Normal breast ductal cells stained more intensely for IGCR-1 than tumor ductal cells. Case 2: The arrow points to area of normal ductal cells in metastatic breast cancer. Metastatic areas localized on left side of sample. (B) IGCR-1 positive normal smooth muscle cells of large arteries compared with IGCR-1 downregulation in smooth muscle cells of leiomyosarcoma. Slides were visualized in light microscopy at 10X.

IGCR-1 expression is maintained in endothelial cells of breast cancer and leiomyosarcoma

Because previously studied molecule in IGCR-1's family, IGPR-1, was shown to have modulating effects on angiogenesis, we wanted to explore the level of IGCR-1 expression in endothelial cells of the tumor microenvironment compared with endothelial cells in normal tissues. As shown before, IGCR-1 is expressed in endothelial cells of normal lung tissues (Figure 5C). Western blot analysis of an umbilical vascular endothelium cell line (HUVECT-TERT) also illustrated positive IGCR-1 protein levels in normal endothelial cells (Figure 9C). However, contrary to the phenomenon of downregulation observed in ductal breast and smooth muscle tumor cells, IGCR-1 showed no evidence of downregulation in endothelial cells of the breast and smooth

muscle tumor microenvironment when compared to those of normal blood vessels (Figure 9A-B). Countless microvessels in leiyosarcomas and breast cancer samples stained positively for IGCR-1 (Figure 9A-B). In addition, endothelial cells of the large arteries in leiomyosarcomas stained positively for IGCR-1 (Figure 9A).

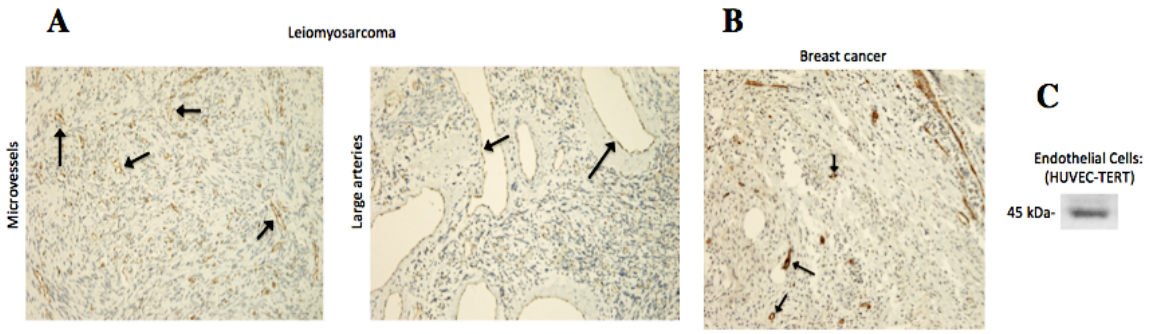


Figure 9. Positive IGCR-1 expression in endothelial cells of the tumor microenvironment and in normal endothelial cell line (HUVEC-TERT). (A) Vast numbers of microvessels and large arteries stained positively for IGCR-1 in leiomyosarcomas (B) Microvessels within metastatic breast tumor environment also indicated positive levels of IGCR-1. (C) IGCR-1 is present in normal umbilical vascular endothelium cell line (HUVEC-TERT). Slides were visualized in light microscopy at 10X.

IGCR-1 expression has an inverse relationship with non-small cell lung cancer staging

Finally, we come to the core of our investigation to determine IGCR-1's potential role as a novel molecule in lung carcinogenesis. In order to elucidate its function in lung carcinogenesis, we wanted to determine the correlation between IGCR-1 expression and the progression of NSCLCs. To do this, we carried out IHC analysis of a lung carcinoma progression tissue microarray consisting of cases from all three subtypes of NSCLCs:

adenocarcinomas, squamous cell carcinomas, and large cell anaplastic carcinomas. Our results showed a gradual decrease of IGCR-1 expression with each progressive stage of carcinoma (Figures 10-12). Non-small cell lung cancer cell line, H3030, also exhibited low expression of IGCR-1 when compared with positive control (HEK-IGCR1) in western blot analysis (Figure 10a). Additionally, endothelial cells of microvessels of lung squamous carcinomas exhibited positive expression of IGCR-1 in a similar fashion as visualized in breast cancer and leiomyosarcoma tissues (Figure 11C).

A) IGCR-1 is downregulated in lung adenocarcinomas

Adenocarcinomas represent the majority of NSCLCs at 38.5% of all lung cancers (Howlader et al, 2010). Hence, we wanted to begin with the results for IGCR-1 expression levels in this specific category of NSCLCs. Results showed significant downregulation of IGCR-1 with each progressive stages (Figure 10b-d). Western blot analysis showed that, compared with the positive IGCR-1 control (HEK-IGCR1), lung adenocarcinoma cell lines (A549 and PC-9) showed lower levels of IGCR-1 (Figure 10a). This is consistent with IHC findings of IGCR-1 being downregulated in breast and smooth muscle tumor cells (Figure 8).

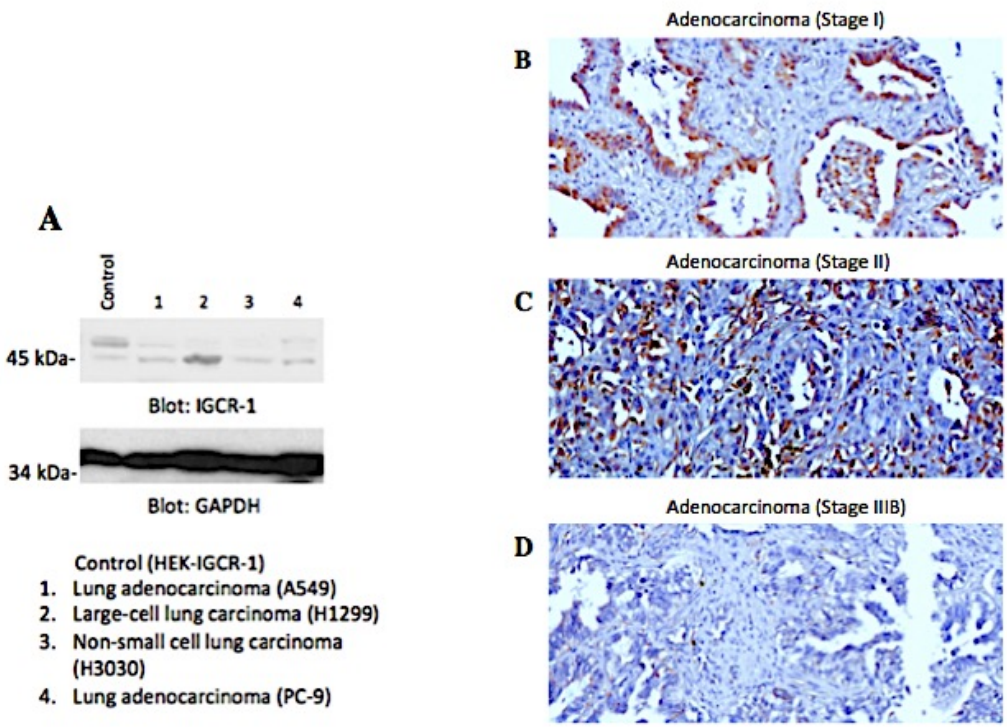


Figure 10. Downregulation of IGCR-1 in later staged lung adenocarcinomas. (A) Cell lysates from human lung adenocarcinoma (A549), large-cell lung carcinoma (H1299), non-small cell lung carcinoma (H3030), and lung adenocarcinoma (PC-9) were subjected to western blot analysis with anti-IGCR-1 and anti-GAPDH antibody as loading control. HEK-IGCR-1 was used for positive control and had a higher molecular weight around 46kDa due to cMyc-tag. (B) IHC analysis showed strong IGCR-1 staining in stage I adenocarcinoma. (C) Stage II indicated decreased staining. (D) No significant staining visualized in stage IIIB adenocarcinoma. Slides were visualized in light microscopy at 10X.

B) IGCR-1 is progressively downregulated in lung squamous cell carcinomas (SCCs)

In a similar way, IGCR-1 is progressively downregulated with later staged lung squamous cell carcinomas (Figure 11). Relative to stage 1 and 2 lung adenocarcinomas (Figure 10b-c), stage 1 and 2B SCCs in IHC analysis expressed lower levels of IGCR-1

(Figure 11a-b). Interestingly, IGCR-1 maintained expression in numerous microvessels of stage IIIA sample SCC (Figure 11c). This is consistent with a previous observation of positive IGCR-1 expression in endothelial cells of breast cancer and leiomyosarcomas (Figure 9a-b).

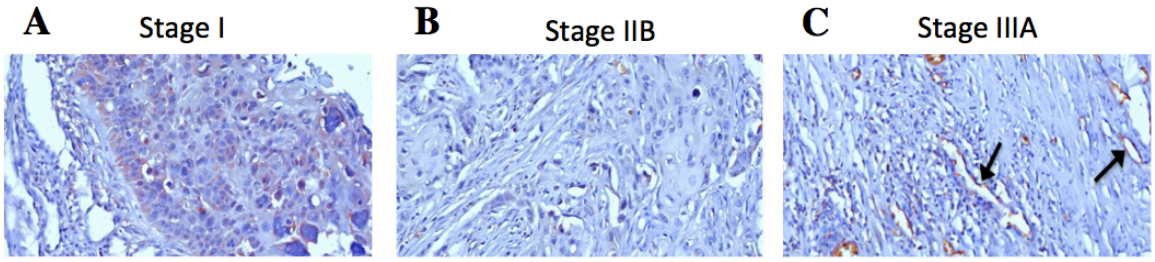


Figure 11. Downregulation of IGCR-1 in squamous cell carcinoma (SCC). (A) An example of a stage I SCC section showing moderate staining of IGCR-1 (B) Insignificant levels of IGCR-1 in a stage IIB SCC sample (C) Low expression of IGCR-1 in a stage IIIA SCC sample, but maintained expression of IGCR-1 in endothelial cells of microvessels. Slides were visualized in light microscopy at 10X.

C) Higher expression of IGCR-1 in early staged large cell anaplastic tumors compared with other NSCLCs and similar downregulation in later staged tumors

Finally, we end with the least common type of lung cancers, large cell lung carcinomas, which make up 2.9% of all lung cancers (Howlader et al, 2010). For this category of NSCLCs, greater levels of IGCR-1 were observed in early stages of the cancer at stage IB and II (Figure 12a-b) compared with early staged lung adenocarcinomas and SCCs (Figures 10-11). More specifically, stage II large cell anaplastic tumor still expressed noticeable levels of IGCR-1 whereas stage II lung adenocarcinomas and stage IIB SCCs did not (Figure 12b, 10c, and 11b). This may correlate with western blot analysis showing greatest relative protein levels of IGCR-1 in

large-cell lung carcinoma cell line (H1299) compared with other NSCLC cell lines

(Figure 10a).

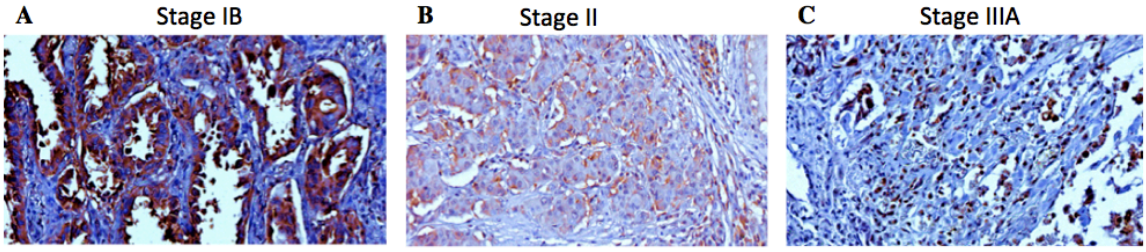


Figure 12. IHC analysis showing higher levels of IGCR-1 in large cell anaplastic carcinoma compared with other NSCLCs and downregulation of IGCR-1 with progressive staging. (A) High level of IGCR-1 expression in stage IB large cell anaplastic carcinoma sample (B) Sample of stage II carcinoma had decreased IGCR-1 levels but still noticeable amounts (C) Low expression of IGCR-1. Intense stains in stage IIIA sample do not bear resemblance to IGCR-1 pattern of staining and are most likely artifacts. Slides were visualized in light microscopy at 10X.

DISCUSSION

Our study demonstrates that IGCR-1 is expressed in lung bronchiole epithelial cells. Its expression, however, progressively trends towards downregulation in non-small cell carcinomas (NSCLCs).

Analysis of bioGPS dataset for IGCR-1 transcript combined with our IHC analysis of mouse and human tissues revealed that IGCR-1 is highly expressed in bronchiole epithelial cells. Moreover, our initial immunoflourescent results indicate that IGCR-1 may be a cell-surface molecule. Its cell surface localization suggests that IGCR-1 could be a cellular adhesion molecule (CAM) with roles in the tumor microenvironment.

Bronchial epithelial cells consist of ciliated and non-ciliated cells that are specifically involved in defending the lungs from toxic substances (Knight and Holgate, 2003). Club cells (Clara cells), the non-ciliated cells of the bronchiole epithelium, aid in forming a protective barrier for the bronchiolar epithelium, metabolizes xenobiotic substances inhaled from the environment, and generates new cells to replenish the epithelium (Komáromy and Tigyi, 1988; Knight and Holgate, 2003). There is a high level of communication between cells of the bronchiole epithelium and cell types outside the epithelium such as stromal cells, fibroblasts, smooth muscle cells, mononuclear cells, lymphocytes, endothelial cells, and myofibroblasts (Thompson et al, 1995; Holgate, 2000). Taken together, the highly specific expression of IGCR-1 in the bronchiolar

epithelium and its cell-surface properties point to its potential roles in mediating communication between a diverse number of cell types.

To develop this thought further, aberrant communication, especially between cells with progenitor functions such as club cells, may then drive carcinogenesis (Crystal et al, 2008). Thus, we propose that insufficient expression of IGCR-1 may cause defective communication between cell types and lead to carcinogenesis. Our findings from IHC and western blot analyses revealed that IGCR-1 expression is significantly reduced or non-detectable in breast cancer, leiomyosarcoma, and three types of later staged NSCLCs. These observations are consistent with the hypothesis that IGCR-1 is an important mediator of cellular communication and subsequent downregulation could cause tumorigenesis. Tumor cells showing downregulation of IGCR-1 compared with normal tissues may suggest that 1) tumor cells selectively mutate to decrease expression of IGCR-1 in order to maintain its survival and eventually achieve metastasis, or 2) genetic or structural mutations that decrease functional levels of IGCR-1 may catalyze tumorigenesis.

Interestingly, our IHC analysis yielded a seemingly paradoxical phenomenon of IGCR-1 expression in the blood vessels surrounding tumors. Although IGCR-1 is consistently downregulated in tumor cells of breast, smooth muscle, and lung cancers, its expression was maintained in the blood vessels that were associated with these tumor samples. This unique expression profile of IGCR-1 in tumor-associated vasculature may suggest that it has a different role in tumor-associated blood vessels than in the tumor cells themselves.

Understanding the tumor microenvironment by exploring the expression and associated function of novel molecules can give us new perspectives and insights on the nature of cancers. Specifically, in our study of IGCR-1, the novel molecule was found to be expressed in lung bronchiole epithelial cells and subsequently regulated in diverse ways depending on cell types in the tumor microenvironment. This complex manifestation of IGCR-1's expression complements the known complexity and heterogeneity in NSCLCs.

The design and development of effective treatment plans for cancer patients, specifically for NSCLC patients, will not be a simple feat. Carcinogenesis involves a host of mutations and aberrant functioning molecular players. Developing treatment plans that simply target one of the many players such as EGFR or VEGF TKIs have proven insufficient, as treated tumors have shown evidence of recurrence due to developed resistance to such therapies. Consequently, there may be a need for adjunct therapies to complement existing therapeutics that target novel molecules in the tumor microenvironment. IGCR-1 is one such molecule that offers potential as a biomarker or target for the development of adjunct therapies to help treat patients with NSCLCs.

Although preliminary in nature, our studies have provided a foundation upon which we can springboard the design of succeeding studies and directed biochemical assays to understand IGCR-1's function in the context of cancer biology. Further investigation of molecules such as IGCR-1 will prove fruitful, as knowledge of novel and unique molecules in the tumomicroenvironment will paint another dimension to our still limited understanding of lung carcinogenesis. This, in turn, could propel innovative

engineering of novel therapies to complement or replace conventional therapeutics that are still largely ineffective at improving the five-year survival rate for NSCLC patients. Ultimately, IGCR-1 may offer direction in the continued search for more effective treatments or diagnostic tools for NSCLC patients, making the effort to fully characterize this novel molecule imperative and worthwhile.

LIST OF JOURNAL ABBREVIATIONS

BMC Cancer	BioMed Central Cancer
CJASN	The Clinical Journal of the American Society of Nephrology
J Biol Chem	The Journal of Biological Chemistry
J Cell Sci	The Journal of Cell Science
Prog Mol Biol Transl Sci	Progress in Molecular Biology and Translational Science

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